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Zur Erklärung der Zweibuchstaben-Codes und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

(54) **Title:** FORMULATION FOR INWARDLY TRANSFERRING NUCLEIC ACIDS INTO EUCARYOTIC CELLS

(54) **Bezeichnung:** FORMULIERUNG ZUR EINSCHLEUSUNG VON NUKLEINSÄUREN IN EUKARYOTISCHE ZELLEN

(57) **Abstract:** The invention relates to a pharmaceutical formulation for inwardly transferring nucleic acids into eucaryotic cells, characterized in that the formulation has a pH value of 6.0 - pH 7,4, and/or an anion concentration of 5 - 100 mmol/l and/or a concentration of 10 - 500 ?mol/l of nonsteroidal antiphlogistics.

(57) **Zusammenfassung:** Die vorliegende Erfindung betrifft eine pharmazeutische Formulierung zur Einschleusung von Nukleinsäuren in eukaryotische Zellen, dadurch gekennzeichnet, dass die Formulierung einen pH-Wert im Bereich von pH 6,0 bis pH 7,4, und/oder eine Anionen-Konzentration im Bereich von 5 bis 100 mmol/l und/oder nichtsteroidale Antiphlogistika mit einer Konzentration im Bereich von 10 bis 500 µmol/l aufweist.

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Cited during the German examination procedure (DE 102 40 418.6)

(1) CalPhos™ Mammalian transfection kit, Handbuch, CLONTECH (14. März 2002), Seite 1-10)

(2) Internetdokument: Transfection, transduction, titer determination & helper virus assay with Neo(+) and Neo(-) vectors, Transfektionsprotokoll (März 1997) Seite 1-9, Internetseite: http://www.usc.edu/hsc/chla/vectorcore/Tran.html)

(3) Buchwald AB u.a. Decoy oligonucleotide against activator protein-1 reduces neointimal proliferation after coronary angioplasty in hypercholesterolemic minipigs (2002) Journal of the American College of Cardiology, Vol.39 (4), Seite 732-738.

I.

Es ist bei Erwägung von Anspruch 1 unklar, ob die Anmelderin für eine Formulierung als Erzeugnispatent Schutz begehrt, oder ob der Schutzbereich auf die Verwendung der Formulierung zur Einschleusung von Nukleinsäuren in eukaryotische Zellen beschränkt sein soll (Verwendungspatent) (siehe Schulte, PatG, 6. Auflage, § 1 Rdn. 187). Im Falle eines Erzeugnispatents ist darauf hinzuweisen, dass die Gegenstände der vorliegenden Patentansprüche zu breit gefasst sind, da offensichtlich nur doppelsträngigen Decoy Oligonukleotide (AP-1, C/EBP u. STAT-1) sowie Antisense-Oligonukleotide (für Caveolin-1 und Folatcarrier (hRTC)) getestet worden sind. Aufgrund der vorliegenden Beschreibungen muss davon ausgegangen werden, dass der Anmelderin zum Zeitpunkt der Anmeldung keine weiteren konkreten Nukleinsäurekonstrukte bzw. Formulierungen zur Verfügung gestanden haben. Damit bestehen begründete Zweifel bezüglich der technischen Ausführbarkeit und somit einer ausreichend deutlichen Offenbarung der Lehre der Anmeldung, da dem fachkundigen Dritten offen bleibt, ob außer den beschriebenen, kurzen Nukleinsäuren, auch große lineare oder zirkuläre DNAs (PCR-Produkte, Plasmide, Vektorkonstrukte) unter den angegeben Bedingungen anmeldungsgemäß zum Erfolg führen und damit vom vorliegenden Schutzbegehren umfasst sind.

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(1)

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CalPhos™ Mammalian Transfection Kit User Manual

PT3025-1 (PR23990)

Release date: 14 March 2002

Catalog #: K2051-1

Store components at –20°C or 4°C as directed.

FOR RESEARCH USE ONLY

Table of Contents

I. Introduction

The ability to introduce exogenous DNA into cultured cells is a powerful tool for molecular and cell biologists. Of the many methods to introduce DNA into mammalian cell cultures, the calcium phosphate method is one of the most widely used because it is inexpensive, simple, and suitable for a range of different cell types (Ausubel *et al.*,1994; Graham & van der Eb, 1973). The CalPhos Mammalian Transfection Kit provides high-quality, pretested reagents suitable for both transient and stable transfections. The kit includes all the reagents necessary to perform 100 transfections in 10-cm plates, or 750 transfections in 35-mm plates.

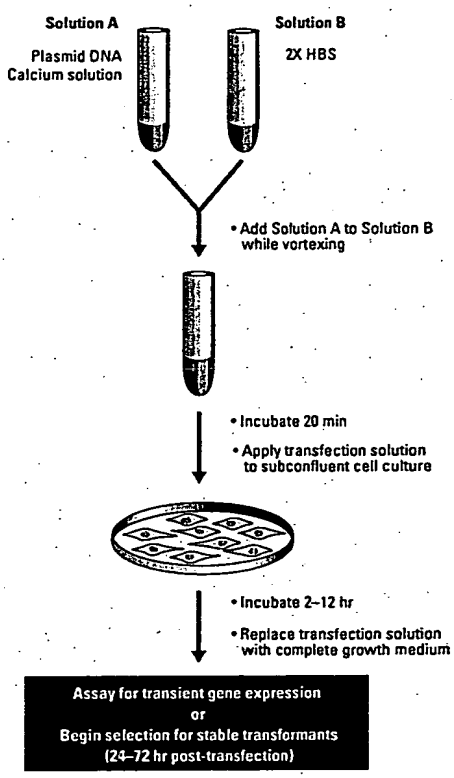

Figure 1. Flow chart for using the CalPhos Mammalian Transfection Kit.

II. List of Components

Store HBS at –20°C; store all other components at 4°C.
The following reagents are sufficient for 100 transfections in 10-cm plates or 750 transfections in 35-mm plates.

- 9 ml **2 M Calcium Solution**
- 2 x 35 ml **2X HEPES-Buffered Saline** (HBS)

We recommend dispensing this buffer into small aliquots, to be stored at –20°C. Avoid multiple freeze-thaw cycles. When an aliquot is in use, store it at 4°C for up to one week.

- 2 x 35 ml **Sterile H_2O**

III. Additional Materials Required

- **Cell culture plates or flasks**
- **Tubes** (12 x 75-mm sterile tubes)
- **Cell culture medium** (appropriate growth medium for mammalian cells in culture)
- **Fetal bovine serum, newborn calf serum, or equivalent** (to supplement the growth medium)
- **Phosphate buffered saline** (PBS; pH 7.4)

	Final Conc.	To prepare 2 L of solution
Na_2HPO_4	58 mM	16.5 g
NaH_2PO_4	17 mM	4.1 g
NaCl	68 mM	8.0 g

Dissolve the above components in 1.8 L of deionized H_2O. Adjust to pH 7.4 with 0.1 N NaOH. Add deionized H_2O to final volume of 2 L. Store at room temperature.

- **1X Trypsin/EDTA** (Life Technologies #25300-054)
- **Plasmid DNA**
  The DNA should be of high quality e.g., double CsCl-banded or column-purified DNA. CLONTECH offers many NucleoBond® Plasmid Purification Kits and cartridges which yield "transfection-grade" plasmids. See Related Products for more information.

IV. CalPhos Mammalian Transfection Protocol

The following protocol is designed for use with adherent cultures growing in **35-mm tissue-culture plates**. If you are using plates, wells, or flasks of a different size, adjust the components in proportion to the surface area of the container you are using. See Appendix B for culture plate conversions.

All steps of the following protocol should be performed in a sterile tissue culture hood.

1. Plate the cells the day before the transfection experiment. The cells should be 50–80% confluent the day of transfection. Generally, we plate 4 x 10^5 cells/35-mm plate.
2. 0.5–3 hr prior to transfection, replace culture medium on plates to be transfected with 2 ml of fresh culture medium per 35-mm plate.
3. For each transfection, prepare Solution A and Solution B in separate sterile tubes.

   Solution A: add components in the following order:

2–4 µg	Plasmid DNA
	Sterile H_2O
12.4 µl	2 M Calcium Solution
100 µl	Total Volume

   Solution B: 100 µl 2X HBS

   Note: To reduce variability when transfecting multiple plates with the same plasmid DNA, prepare master solutions of Solutions A and B sufficient for all plates.
4. Carefully and slowly vortex Solution B while adding Solution A dropwise. (Alternatively, blow bubbles into Solution B with a 1-ml sterile pipette and an autopipettor while adding Solution A dropwise.)
5. Incubate the transfection solution at room temperature for 20 min.
6. Gently vortex transfection solution and then add solution dropwise to culture plate medium. (Add 200 µl of transfection solution per 35-mm plate.)
7. Gently move plates back and forth to distribute transfection solution evenly. (Do not rotate plates as this will concentrate transfection precipitate in the center of the well or plate.)
8. Incubate plates at 37°C for 2–12 hr in a CO_2 incubator.
9. Remove calcium phosphate-containing medium and wash cells with medium or 1X PBS.
10. Feed plate with 2 ml fresh complete growth medium and incubate at 37°C until needed for assay.
11. Assay for transient gene expression or start selection for stable transformants 24–72 hr post-transfection.

V. Troubleshooting Guide

A. Low Transfection Efficiency

- Poor precipitate formation

  Solution: Addition of the calcium/DNA (Solution A) to the 2X HBS (Solution B) should be performed dropwise and with continuous mixing. Adding Solution A too quickly or with too little mixing can result in a poor precipitate.

- Poor quality DNA

  Solution: The A_{260}/A_{280} ratio of the plasmid DNA should be ≥1.7.

- pH not optimal

  Solution: The pH of the HBS should be between 7.05 and 7.12. However, during prolonged storage, the pH of the solution may change; therefore, use the transfection kit within the shelf life indicated on the accompanying Product Analysis Certificate (PAC).

B. Variable Transfection Efficiency in Experiments

There will always be some variability in transfection efficiencies. We recommend performing transfections in triplicate to minimize the variability.

- Variable cell density

  Solution: Keep cell density constant after optimizing transfection procedures. Generally we use cultures that are 50–80% confluent at the time of transfection.

- Suboptimal cell growth

  Solution: Keep cells healthy in culture. Cells should be in mid-log phase growth when plated for transfection. Transfection efficiencies may decrease for cell lines that have been passaged for too many generations.

VI. References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1994) In *Current Protocols in Molecular Biology* (Greene Publishing Associates and John Wiley & Sons, Inc., NY) Vol. 1, Ch. 9.

Freshney, R. I. (1993) *Culture of Animal Cells,* Third Edition (Wiley-Liss, NY).

VII. Related Products

For the latest and most complete listing of all BD Biosciences Clontech products, please visit **www.bdbiosciences.com**

• Clonfectin™	8020-1
• Living Colors™ Fluorescent Protein Reporter Vectors	many
• Tet-On™ and Tet-Off™ Expression Systems and Vectors	many
• Great EscAPe™ Secreted Alkaline Phosphatase (SEAP) Vectors and Kits	many
• β-galactosidase Vectors and Kits	many
• IRES Expression Vectors	many
• Retro-X™ Expression Vectors	many
• Mammalian MATCHMAKER Two-Hybrid Assay Kit	K1602-1
• NucleoBond® Plasmid Kits	
Midi Kit	K3002-1, -2, -3
Maxi Kit	K3003-1, -2, -3
AX Tips	many

Appendix A: Optimization of Transfection

The efficiency of a mammalian cell transfection is primarily dependent on the host cell line used. Optimization of the transfection parameters for each cell type is crucial to obtaining consistently successful transfections. Therefore, for each cell type you plan to use, perform preliminary experiments to determine the optimal: 1) cell density; 2) amount and purity of DNA; and 3) transfection incubation time.

For the preliminary experiments, the host cell line can be transfected with a reporter expression vector, such as pCMVβ (#6177-1) or pSEAP2-Control Vector (#6052-1). The success of the transfection can then be estimated by assaying for β-galactosidase or secreted alkaline phosphatase. Once the transfection parameters have been optimized, they should be kept consistent from one experiment to the next to obtain reproducible results.

The following is a general guideline for optimizing the transfection parameters. To optimize transfection parameters, it is best to perform a series of small-scale transfections. This can be done conveniently in 12-well or 6-well plates.

To optimize cell density: keeping all other parameters constant, plate host cells in individual wells of a 6-well plate at varying densities (e.g., 5×10^4, 1×10^5, 2×10^5, 4×10^5, 8×10^5). 24–72 hours post-transfection, assay for reporter gene (SEAP or β-galactosidase) activity. Record results. Repeat the experiment once or twice to account for day-to-day variation. Choose the density with the highest reporter gene activity.

The other parameters can be optimized in much the same way. Hold all other variables constant while varying the parameter you are testing. Transfection incubations should be maximal at 2–16 hours using the CalPhos Mammalian Transfection Kit. You may want to try incubation times from 1–18 hours for optimization. After transfections have been optimized, scale-up or scale-down as necessary for the size of culture plate you are using (see Appendix B for a table of conversions).

Appendix B: Culture Plate Conversions

TABLE II. CULTURE PLATE CONVERSION

Size of Plate	Growth Area (cm^2)	Relative Area*	Recommended Volume
96 well	0.32	0.04 X	200 µl
24 well	1.88	0.25 X	500 µl
12 well	3.83	0.5 X	1.0 ml
6 well	9.4	1.2 X	2.0 ml
35 mm	**8.0**	**1.0 X**	**2.0 ml**
60 mm	21	2.6 X	5.0 ml
10 cm	55	7 X	10.0 ml
Flasks	25	3 X	5.0 ml
	75	9 X	12.0 ml

* Relative area is expressed as a factor of the growth area of a 35-mm culture plate.

Notes:

ChildrensHospital LosAngeles

Transfection, Transduction,Titer determination & Helper virus assay with Neo(+) and Neo(-) vectors

Updated 3/97

Compiled by Lars BrandŽn# & Karen Pepper$

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This is a detailed protocol designed for those who have limited transfection experience.

Transfection and transduction with Neo(+) vector

Note: In this protocol GPE cells are the transfected cell line and PA317 cells are the final producer cell line.

1. Thaw GPE cells and add D10 up to 10 ml.

2. Spin cells 3 minutes at 1500 rpm, room temp. in bench centrifuge.

3. Aspirate of media and resuspend in 10 ml D10.

4. Plate 10 ml GPE cell suspension on 2 100mm plates, add 5 ml more of D10.

5. Grow overnight.

6. Aspirate D10 media off GPE cells. Wash cells with 10 ml Hanks and then add 1X trypsine(0.5 ml/100 mm plate). Remove trypsine immediately and put in incubator for 5 min., then add 10 ml full media(D10), vigorously aspirate cells from plate. Take 15 μl and add 15 μl trypan blue to it for counting the cells. Plate 5x10*5 cells/plate in 10 ml full media(D10)
To determine the number of cells/ml use following equation:
X/4x2x10*4=cells/ml
2=Dilution factor
X=number of cells in the four quadrants in a cell counter.

7. Incubate cells 4-24 h.at 37C, 5% CO2, before transfection.

8. Dilute 5 μg DNA up to 100 μl with HBS. Put in spin-X(COSTAR), 0.45 μm is best to sterilize.

9. In a 3 ml polystyrene tube, mix 70 μl transfection reagent (DOTAP) with 180 μl sterile HBS. Add it to the DNA solution, mix and incubate at room temperature for 10 min.

10. Aspirate media off cells.

11. Add 2x5 ml of D10 media to DNA/transfection mix. You have to use a 5 ml pipette for the 3 ml tubes, because the 10 ml will not fit. Add to plate. Incubate on cells for 24 h. Thaw PA317 cells to have ready for next steps.

12. Next day aspirate media off GPE plates and wash with Hanks. Add fresh D10 full media. Incubate overnight.*Plate PA317 cells for the next day, 5x10*5/plate

13. Next day collect supernatant from both GPE plates. *Add to PA317 cells which were plated the day before at 5x10*5/plate in D10 full media.(1000) To GPE cells left over(back-up), add media containing G418 antibiotic.Let GPE cells select for 7-10 days See below for times.

9:00am: Collect 10 ml of supernatant off transfected GPE cells, use 10 ml syringe + 0.45 μm syringe filter. Add supernatant to one plate of PA317. Add 100 μl of 800 ug/ml polybrene. Swirl plate. Incubate until noon.

Noon: Aspirate off supernatant from PA317 cells --->optional!!(and take 2nd plate of GPE cells, collect supernatant as before using a 10 ml syringe), add to PA317 plate + 100 μl 800 ug/ml polybrane. Incubate until 5:00pm

5:00pm: Aspirate supernatant off PA317 plate. Add 10 ml fresh D10. (without G418) Incubate 2 days.

At same time plate GPE cells for G418 selection

9:00 Take first plate of GPE used, trypsinize cells, add 10 ml D10 media and plate it on two plates, 5 ml each. Then add 5 ml of 1xHXM media(final is 1/2xHXM). add 100 μl /plate of 50 mg/ml G418 (which is stored at -20C).

Noon --->Optional!!Do same as above for 2nd plate of GPE cells. Now you will have 4 plates for G418 selection.

---

14. 1-3 days later, depending on when confluent, PA317 plate needs to be split and trypsinize. Make 10-fold serial dilutions of cells.
9/10
1/10
1/100
1/1000
Plate in D10(total volume 10 ml)+G418
9/10 plate will become confluent, then use as pool, collect cells and then freeze

. 15. When plates develops colonies, choose plates with isolated colonies and isolate with glass-rings and plate1 colony/well in 12 well dish, grow to confluency in G418, then plate in 100 mm dish. Collect supernatants from dishes and assay titer, and at the same time cryo preserve cells.

16. GPE plates(have 4/transfection) after 7-10 days. Because there will be so few cells, trypsinize and plate all cells in 1 or two plates. Grow to confluency. Change D10 full media(no G418), collect supernatant and transfect PA317 cells, as previously(If needed). Cryo preserve cells.

Transfection and transduction with Neo(-) vector

1. Thaw GPE cells and add D10 up to 10 ml.

2. Spin cells 3 minutes at 1500 rpm, room temp. in bench centrifuge.

3. Aspirate of media and resuspend in 10 ml D10.

4. Plate 10 ml GPE cell suspension on 2 plates, add 5 ml more of D10.

5. Grow overnight.

6. Aspirate D10 media off GPE cells. Wash cells with 10 ml Hanks and then add trypsine(0.5 ml/100 mm plate). Remove trypsine immediately and put in incubator for 5 min., then add 10 ml full media (D10), vigorously aspirate cells from plate.
Take 15 μl and add 15 μl trypan blue to it for counting the cells.
Plate 5x10*5 cells/plate in 10 ml full media(D10) on 4 plates.
To determine the number of cells/ml use following equation:
X/4x2x10*4=cells/ml
2=Dilution factor
X=number of cells in the four quadrants in a cell counter.

7. Incubate cells 4-24 h.at 37C, 5% CO2, before transfection.

8. Dilute 5 μg of your vector DNA and 5 μg of pSv2Neo up to 100 μl with HBS. Put in spin-X (COSTAR), 0.22 μm or .45um Set up 1 tranfection perGPE plate.(Or use 9:1 ratio)

9. In a 3 ml polystyrene tube, mix 70 μl transfection reagent with 180 μl sterile HBS. Add it to the DNA solution, mix and incubate at room temperature for 10 min.

10. Aspirate media off cells.

11. Add 2x5 ml of D10 media to DNA/transfection mix. You have to use a 5 ml pipette for the 3 ml tubes, because the 10 ml will not fit. Add to plate. Incubate on cells for 24 h.

12. Next day aspirate media of GPE plates and wash with 10 ml Hanks. Add fresh D10 media and 100 μl G418 (50 mg/ml stock).

13. Incubate until GPE cells are almost confluent. If nessesary change media after 5-7 days.

14. Thaw PA317 cells to have ready for next steps.

15. When GPE cells look almost confluent aspirate off media and add fresh D10(no G418) and incubate overnight.

Plate PA317 5x10*5 cells/plate in 10 ml full media(D10). Incubate overnight.

16. Next day collect supernatant from GPE plates. *add to PA317 cells which were plated the day before at 5x10*5/plate in D10 full media. Look below for times.

Times

9:00am: Collect 10 ml of supernatant off transfected GPE cells, use 10 ml syringe + 0.45 μm syringe

filter. Add supernatant to pl[redacted]f PA317. Add 100 µl of 800 ug/ml polybr[redacted]. Swirl plate. Incubate until 11:00(2 h. incubation at least).

11:00: Aspirate off supernatant from PA317 cells. Repeat with the three other GPE plates.(Or can do repeated infection with supernatant from 1 plate of transfected GPE over a few days)

5:00pm Aspirate supernatant off PA317 plate. Add 10 ml fresh D10. (without G418)
Incubate 2 days.(or until confluent)
Trypsinize GPE cells and freeze them down(as a back up).

---

17. 1-3 days later, depending on when confluent, PA317 plate needs to be split and trypsinize. Make 10-fold serial dilutions of cells.
9/10
1/10
1/100
1/1000 - 1/100,000
Plate in D10(total volume 10 ml)(NO G418)

18. 9/10 plate will become confluent, then use as pool, collect cells and then freeze. When plates develops colonies, choose plates with isolated colonies and isolate with glass-rings and plate1 colony/well in 12 well dish, grow to confluency (NO G418), then plate in 100mm dish with D10. Collect supernatants from dishes and assay for presence of retroviral vector using PCR or other method., and at the same time cryo preserve cells.

Media

D10 full media:(Lasts about 2 months(L-glut. potency expires)
DMEM, high glucose: 450 ml
FCS (or NCS): 50 ml
L-glut: 5 ml
Pen/Strep: 2.5 ml

HBS:
20 mM Hepes
150 mM NaCl pH 7.4

3T3 Media:
DMEM, high glucose: 450 ml
NewbornCS: 50 ml
L-glut 100x from Sigma : 5 ml
Pen/Strep: 2.5 ml

HXM: (routine growth of GPE cells only)
FCS: 50 ml
Pen/Strep : 5 ml
10 mg/ml Hypoxanthine : 0.75 ml
10 mg/ml Xanthine : 12.5 ml
10 mg/ml mycophenolic acid: 1.25 ml
HCl : 100-150 µl
DMEM, high glucose : 450 ml

Freezing media:
FCS : 80%
DMSO : 20%

G-418
Dilute in 0.1 M Hepes buffer to final concentration of 50 mg/ml and store at -20C.

Polybrene(Hexadimethrine Bromide) Sigma cat.#H-9268
Hank's balanced salt solution: Irvine Scientific cat.#9228
Trypsin/EDTA: Irvine scientific cat.#9340
Transfection reagent: Boerhinger Mannheim cat.#1202375
Pen/Strep Irvine Scientific cat.#9366
Geneticin(G-418 sulfate) GIBCO cat.#860-1811

Titering Neo(+) supernatants

1. Make 1 six-well tray for each supernatant to be titered.

2. 3T3 cells should come from a log phase plate, not a confluent plate.

3. Plate 3T3 fibroblasts in 6 well trays at 2.5x10*4 cells/well in 2 mls of media.(-ie make cell suspension at 1.25x10*4 and put 2 mls in each well).

4. Grow cells overnight

5. Next day collect virus-containing supernatant(s) to be titered from producer cells. Using a 10 ml syringe filter through 0.45 μm syringe filter to remove cells or spin at 3000 rpmx5 min.

6. Put 2.5 mls of each cell-free viral supernatant into tubes. (Undiluted)

7. Put 2.5 mls D10 into 4 tubes for each 6 well tray for serial dil.:
10*(-2), 10*(-4), 10*(-5), 10*(-6).

8. Serially dilute virus supernatant. Make sure to use a new tip for each tube.
Tube#-------Final dilution--------Media----------Add
1----------Undiluted----------2.5 ml filtered sup.---0-
2----------100x(10*-2)---------2.5 ml 3T3 media-----25 μl from #1
3----------10 000(10*-4)--------2.5 ml 3T3 media-----25 μl from #2
4----------100 000(10*-5)-------2.5 ml 3T3 media-----280 μl from #3
5----------1 000 000(10*-6)------2.5 ml 3T3 media-----25 μl from #3

9. Remove media from fibroblasts in wells of 6 well trays, plated yesterday. Add 1.0 ml of each dilution of viral containing supernatant to wells.

10. Add to 6 well plates like this:
(#1)-Undiluted
(#2)-10*-2
(#3)-10*-4
(#4)-10*-5

(#5)-10*-6
(#6)-Control*

*Control may be either no virus added(to check G418 killing) or no G418(to check cell growth)

11. Add 20 µl of 800 µg/ml polybrane to every well+control well.

12. Incubate for 2h. at 37ûC 13. Add 1.0 ml additional D10 to each well. Incubate 24h.

14. After 24h., remove media. Add 2ml of D10 with 0.5 mg/ml G418(final concentration)

15. After 7-10 days,check for G418 selection and colony formation, then stain cells MeOH-methylene blue: (0.1g methylene blue/60 ml MeOH)

16. Wash plates 1x with Hank's

17. Add 1 ml stain solution.

18. Incubate att R/T 1-5 min.

19. Dip in water 5x. Dry upside down. Count colonies.

Titering Neo(-) vectors

When you use this method it is a good idea to use the same primers for both the standard and the unknown sample beacause of the difference in efficiency between different primer pairs.

Day 0 Plate 3T3 cells in 12-well plates at 10*4 cells/well. One plate is used for eact vector to be titered. In addition, one plate is used for a control vector which titer is known. The control vector is used as a reference.

Day 1 Infections are done at different dilutions:

1x(undiluted supernatant)
1:2
1:5
1:10
1:50
1:100
1:500
1:1000

The supernatant is diluted with 3T3 medium. The dilutions are done serially, as follows.

Undiluted
1:2
1:5
1:10
1:50
1:100
1:500

1:1000
1:5000
1:10000

1.0 ml of each dilution is plated in each well. Polybrene is added at a final concentration of 8 µg/ml. The plates are incubated for 4 h. The virus containing medium is discarded. The cells are washed 1x with Hanks. Add 1 ml of fresh 3T3 medium to the wells.

When the wells are confluent they are trypsinized. The cells of each well are taken individually into 1.5 ml microcentrifuge tubes. Then whole cell lysates are prepared for PCR.

Preparation of whole cell lysates for PCR

1. Spin down the cells at 14 000 rpm for 30 seconds.

2. Discard the supernatant.

3. Suspend the cells in 1.0 ml of Hanks or PBS

4. Spin again for 30 seconds.

5. Discard the supernatant

6. Resuspend the cell pellet with 50-100 µl whole cell lysate buffer containing proteinas K.

7. Incubate at 55C for 1-2 h (or at 37C o/n)

8. Boil the tubes for 10 minutes in order to inactivate the proteinas K.

9. Use 2-5 µl of the lysate from each sample for PCR

Whole cell lysate buffer:

50 mM KCl
10 mM Tris-HCl (pH 8.3)
1.5 mM MgCl2
0.1 mg/ml gelatin
0.45% NP40
0.45% Tween 20

Autoclave and aliquot in 1.0 ml aliquots. Store at -20C.
When used add fresh proteinas K, 3.0 µl of 10mg/ml solution per 100 µl buffer.

Helper virus test

1. Plate your producer cells 24 h. prior to the plating of the 3T3 cells.

2. Change medium on producer cell plates and incubate 24 h.

3. Plate 3T3 cells in 6 well plate at 10*4 cells/well. Use 3T3 medium. The cells should be taken from a plate in log phase. Incubate 24 h.

4. Plate PA317 cells in 6 well plate at 104 cells/well. The cells should be taken from a plate in log phase. Use D10 medium.This plate is for the positive control in the PCR reaction.Incubate 24 h.

5. Collect supernatant from producer cell plates and filter through 0.45 μm filter to remove cells.

6. Infect 3T3 cells with 2 ml supernatant .

7. Add 40 μl polybrene (800 μg/ml stock) to every well.

8. Incubate 2 h. at 37C.

9. Remove media and repeat 2x.

10. Incubate for 4 days at 37C.

11. Trypsinize cells.

12. Spin down cells at 14000 rpm for 30 s.

13. Discard the supernatant.

14. Suspend the cells in 1 ml of Hanks or PBS.

15. Spin again for 30 s.

16. Discard the supernatant.

17. Resuspend the cells with 500 μl whole cell lysate buffer containing proteinase K,RNase & Spermidine

18. Incubate at 55C for 1-2 h. (or at 37C o/n)

19. Boil the tubes for 10 min. in order to inactivate the proteinas K.

20. Extract with phenol/chloroform.

21. Ethanol precipitate samples.

22. Resuspend in 200 μl 1xTE.

PCR reaction:

34 μl DNA
5 μl 10xPCR buffer
1 μl each of Env 1 & 3 primers
10 μl 2mM dNTP
0.5 μl Taq

Cycle parameters:
1 94C-5 min.
2 60C-2 min
3 72C-2 min
4 94C-1 min
2,3 & 4 x30.

Whole cell lysate buffer for 3T3 test:
50 mM KCl
10 mM Tris-HCl (pH 8.3)
1.5 mM MgCl2
0.1 mg/ml gelatin
0.45% NP40
0.45% Tween 20
Autoclave and aliquot in 1.0 ml aliquots. Store at -20C.
(When used add fresh proteinas K, 10.0 μl of 10mg/ml solution per 500 μl buffer. 2 μl RNase/500 μl buffer)
15 μl 0.1M spermidine/500 μl buffer

PCR primers
Env 1: 5'-ACC TGG AGA GTC ACC AAC C-3'
Env 3: 5'-TAC TTT GGA GAG GTC GTA GC -3'

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Decoy Oligodeoxynucleotide Against Activator Protein-1 Reduces Neointimal Proliferation After Coronary Angioplasty in Hypercholesterolemic Minipigs

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OBJECTIVES We sought to demonstrate, in an appropriate animal model, that co-medication with a transcription factor-blocking agent limits restenosis after percutaneous transluminal coronary angioplasty (PTCA).

BACKGROUND Enhanced synthesis in the vessel wall of endothelin-1 (ET-1), a powerful co-mitogen for vascular smooth muscle cells, appears to be one mechanism that promotes restenosis after PTCA. Deformation-induced expression of prepro-ET-1 is governed by the transcription factor, activator protein-1 (AP-1).

METHODS An anti-AP-1 decoy oligodeoxynucleotide (dODN) strategy was devised in which the dODN-containing solution (20 nmol) was administered locally through a Dispatch catheter into the coronary arteries of hypercholesterolemic minipigs at the time of PTCA (AVE-GFX stent).

RESULTS Treatment with an AP-1 dODN, mimicking the consensus binding site of the transcription factor, significantly reduced neointimal formation in the coronary arteries of hypercholesterolemic minipigs ($n = 10$ to 12), compared with vehicle-treated coronary arteries, after four weeks of follow-up (neointimal area 2.64 ± 0.33 vs. 4.81 ± 1.04 mm^2 [mean ± SEM]; $p < 0.05$). This effect was maintained after eight weeks (neointimal area 2.04 ± 0.22 mm^2; $n = 3$) and correlated with a reduction in both nuclear translocation of AP-1 and ET-1 synthesis in the vessel wall 48 h after PTCA ($n = 4$). In contrast, an AP-1 mutant dODN, to which the transcription factor does not bind, showed no effect on neointimal formation at either time point ($n = 3$ to 7). Moreover, a consensus dODN directed against CCAAT/enhancer binding protein (C/EBP), another deformation-sensitive transcription factor, did not significantly affect neointimal formation after four weeks ($n = 3$).

CONCLUSIONS These findings demonstrate the feasibility, efficacy and specificity of the anti-AP-1 dODN approach to the treatment of restenosis, which principally but not exclusively targets deformation-induced ET-1 synthesis in the vessel wall. Provided that these findings can be extrapolated to the situation of patients with coronary artery disease, the observed extent of the inhibitory effect of the AP-1 dODN treatment suggests that this co-medication may greatly reduce the incidence of in-stent restenosis. (J Am Coll Cardiol 2002;39:732–8)

The implantation of stents has substantially reduced the rate of restenosis after percutaneous transluminal coronary angioplasty (PTCA) (1,2). However, in 10% to 40% of patients, restenosis still occurs, resulting in revascularization of the target lesion in up to 20% of vessels accommodating ≥3.0-mm stents, and even higher rates in smaller arteries and long lesions (3,4). Thus, restenosis contributes substantially to morbidity and the costs of treating coronary artery disease. Restenosis after coronary artery stent implantation is characterized by extensive neointimal proliferation of vascular smooth muscle cells (SMCs) and the formation of extracellular matrix (5–7). Possible mechanisms resulting in this change in SMC phenotype, from a contractile to a synthetic state, appear to be related to an increased synthesis of endothelin-1 (ET-1) in the vessel wall. Thus, enhanced synthesis of ET-1 has been demonstrated to occur after pressure trauma to the vessel wall in experimental models and in humans (8,9).

Previous work from our group has shown that this effect occurs at the level of transcription of the prepro-ET-1 gene (ppET-1) and that a decoy oligodeoxynucleotide (dODN) directed against the transcription factor activator protein-1 (AP-1) inhibits both ppET-1 expression and ET-1 synthesis in cultured endothelial cells exposed to mechanical deformation, as well as in isolated, perfused, endothelium-intact blood vessels in response to a supraphysiologic increase in perfusion pressure (10). Although there are many genes whose expression may be affected by this transcription factor, only a few, such as ppET-1, are sensitive to deformation, and there are only a few in which AP-1 functions as an essential component of the transcription initiation complex.

To verify the therapeutic potential of this dODN approach, we have investigated whether a single administra-

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Abbreviations and Acronyms

AP-1 = activator protein-1
C/EBP = CCAAT/enhancer binding protein
dODN = decoy oligodeoxynucleotide
ET_A = endothelin A
ET-1 = endothelin-1
PBS = phosphate-buffered saline
ppET-1 = prepro-endothelin-1
PTCA = percutaneous transluminal coronary angioplasty
SMC = smooth muscle cell

tion of AP-1 consensus dODN at the time of PTCA can limit neointimal proliferation in a coronary stent angioplasty model in hypercholesterolemic minipigs.

METHODS

Decoy ODN technique. Double-stranded dODN (0.4 mmol/l) was prepared from complementary single-stranded phosphorothioate-bonded ODN obtained from Eurogentec (Köln, Germany), by melting it at 95°C for 5 min, followed by a cool-down phase of 3 to 4 h at an ambient temperature. After that, the solutions were split into 50 μl aliquots and frozen at −80°C until further use. The efficiency of the hybridization reaction was verified with 2.5% agarose gel electrophoresis and usually exceeded 98%. The sequences of the dODN for AP-1, CCAAT/enhancer binding protein (C/EBP) and the corresponding mutant dODN were as follows (underlined letters denote phosphorothioate-bonded bases; bold letters denote the core binding sequence for the transcription factor; and italic letters denote the mutated bases; cons = consensus dODN; mut = mutant dODN):

AP-1cons: 5′-CGCTTGATGACTCAGCCGGAA-3′
3′-GCGAACTACTGAGTCGGCCTT-5′
AP-1mut: 5′-CGCTTGAT*TACTTA*GCCGGAA-3′
3′-GCGAACTAATGAATCGGCCTT-5′
C/EBPcons: 5′-TGCAGATTGCGCAATCTGCA-3′
3′-ACGTCTAACGCGTTAGACGT-5′
C/EBPmut: 5′-TGCAGA*GACTAGTCTCTGCA*-3′
3′-ACGTCTCTGATCAGAGACGT-5′

Before infusion, an aliquot of the dODN solution (50 μl) was defrosted and diluted in 10 ml phosphate-buffered saline (PBS; 2 μmol/l final concentration). As buffer control (vehicle), a corresponding volume of TEN (Tris, EDTA and Nacl) buffer (10 mmol/l Tris-HCl, 1 mmol/l EDTA and 150 mmol/l NaCl, at pH 7.5) was diluted in 10 ml PBS.

Animal model. Animal experiments were done in accordance with the guidelines of the local Committee on Animal Welfare and approved by the local authorities. The experimental model has been described in detail previously (11,12). Briefly, in 22 minipigs under general anesthesia, PTCA was performed through a carotid artery. Per animal, two or three coronary artery segments of a slightly smaller diameter (0.3 to 0.5 mm less) than the 3.0-mm stent-carrying balloon were chosen for treatment, as indicated subsequently. This resulted in placement of stents more distally in some vessels and more proximally in other vessels, depending on their size. Operators had no knowledge of the treatment given to a specific vessel segment (dODN or vehicle). Before PTCA, 0.2 mg of intracoronary nitroglycerin was administered. Stents (AVE-GFX, Medtronic, Düsseldorf, Germany) 12 mm in length were implanted at 16 atm of pressure for 20 s. The balloons were withdrawn, and a Dispatch catheter (Interventional Techniques, San Diego, California, through Cardiologic, Munich, Germany) was advanced into the stented segment and inflated to 6 atm, and infusion of the dODN solution or vehicle was started.

The Dispatch catheter has an inner lumen allowing for peripheral perfusion during inflation. Flow through this inner lumen was verified in 5-min intervals. On inflation, a helical-like–shaped balloon creates a space between the vessel wall and the catheter, into which there is drainage through multiple end holes of the infusion line. There is a slow, distal run-off into the artery, allowing for continuous drug infusion. After the desired volume (10 ml at 0.5 ml/min; corresponding to a total amount of 20 nmol dODN) was infused, the balloon was deflated and withdrawn, the carotid artery was ligated, and the neck wound was closed. The animals were returned to their cages until after the planned follow-up period (see subsequently), when they were euthanized.

Morphometric analysis. After four or eight weeks of follow-up, a thoracotomy under deep anesthesia was performed, and the animals were euthanized by an overdose of barbiturate. The hearts were excised and immediately perfused with PBS at a pressure of 100 mm Hg, followed by perfusion-fixation with buffered 4% formaldehyde (1,000 ml). The angioplasty segments were then excised and embedded in methylmethacrylate. Three sections per 12-mm segment were subsequently analyzed morphometrically using a digital microscopic video camera and the Image Pro software (version 2.0, Media Cybernetics, Silver Spring, Maryland). Areas of the lumen, neointima, media and adventitia, as well as neointimal thickness over each stent strut, were measured. Penetration of each strut into the vessel wall was graded using a modified injury score, as originally described by Schwartz et al. (13), where injury ranged in increments from 1 (superficial) to 4 (into the adventitia). The investigator had no knowledge of the treatment of the segments during morphometry.

Endothelin-1 immunohistochemistry. For immunohistochemical analysis, the angioplasty segments were excised immediately after euthanasia 48 h after PTCA; the stents were removed; and a 3-mm portion was fixed in formaldehyde and embedded in paraffin. The remaining portion was deep-frozen in liquid nitrogen for subsequent electrophoretic mobility shift analysis. Of the paraffin blocks, 5-μm-thick sections were cut and mounted on siliconized

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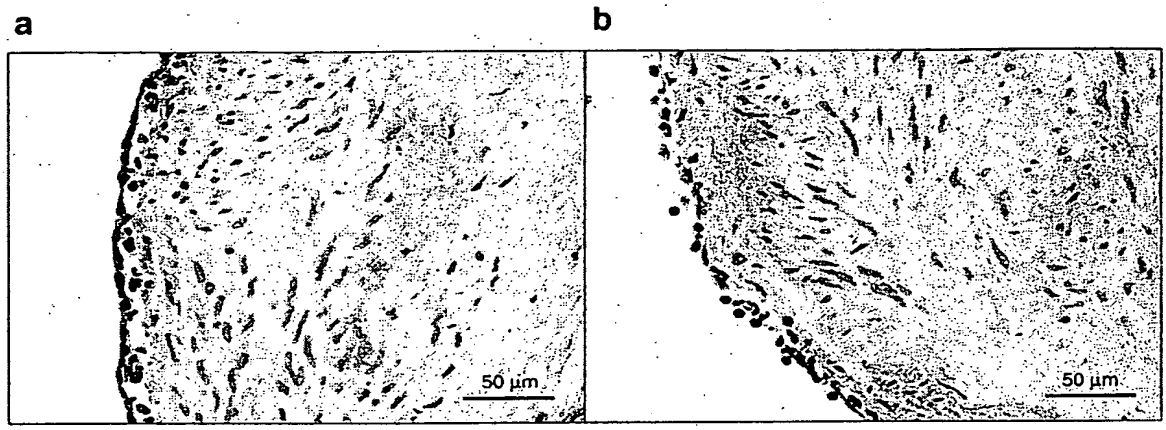

Figure 1. Immunohistochemical detection of endothelin-1 (ET-1) in the coronary arteries of the same animal treated with (a) vehicle or (b) the activator protein-1 (AP-1) consensus decoy oligodeoxynucleotide (dODN) 48 h after percutaneous transluminal coronary angioplasty. Positive immunoreactivity for ET-1, both in the periluminal cell layers and in the media, is markedly reduced in the AP-1 consensus dODN-treated artery (original ×400, before 00% reduction). The results shown are representative of those obtained with four different animals.

slides. After deparaffinization and dehydration, the sections were first stained with hematoxylin, followed by overnight incubation at 4°C with a mouse monoclonal anti-ET-1 antibody (Dianova, Hamburg, Germany) at a dilution of 1:250. For detection of the bound primary antibody, the Histostain Plus kit (Zymed, San Francisco, California) was used, comprising a biotinylated goat–anti-mouse, streptavidin-peroxidase conjugate and the chromogenic substrate 3-amino-9-ethylcarbazol (AEC), yielding a red deposit.

Electrophoretic mobility shift analysis. Nuclear extracts from the coronary artery segments were prepared as described previously for rabbit blood vessels (14). The double-stranded gel shift oligonucleotides (Santa Cruz Biotechnology, Heidelberg, Germany) for AP-1 and C/EBP (sequences identical to AP-1cons and C/EBP dODN, as described earlier) were end-labeled with (γ-^{32}P)adenosine triphosphate by using the 5′ end-labeling kit from Amersham Pharmacia Biotech (Freiburg, Germany). Typically, the binding mixture contained 5 μg of nuclear extract, 20,000 cpm of the ^{32}P-labeled oligonucleotide probe (0.5 ng), 1 μg poly(d[I-C]) and 1.33 mmol/l of DL-dithiothreitol in a total volume of 15 μl binding buffer.

Treatment groups and statistical analysis. Angioplasty of three coronary arteries per animal was performed in six minipigs, with a follow-up period of four weeks (comparisons of vehicle, AP-1 consensus dODN and AP-1 mutant dODN, n = 3; comparisons of vehicle, C/EBP consensus dODN and C/EBP mutant dODN, n = 3), and of two coronary arteries per animal in 16 minipigs, with a follow-up period of 48 h (comparison of vehicle and AP-1 consensus dODN, n = 4), four weeks (comparison of vehicle and AP-1 consensus dODN, n = 5; AP-1 consensus dODN vs. AP-1 mutant dODN, n = 4) or eight weeks (comparison of AP-1 consensus dODN and AP-1 mutant dODN, n = 3).

Statistical analysis. The results are expressed as the mean value ± SEM, with n referring to the number of coronary arteries analyzed per treatment group, regardless of comparisons made in individual animals. Statistical analysis using InStat software, version 3.0 (GraphPad, San Diego, California) was performed by Kruskal-Wallis one-way analysis of variance, followed by Dunn's post test for selected groups or the unpaired *t* test with the Welch correction, as appropriate (comparisons of three or two treatment groups, respectively), with $p < 0.05$ considered as statistically significant.

RESULTS

Serum cholesterol level. Animals were fed a standard diet supplemented with 2% cholesterol and 10 g/day of sodium cholate for at least eight weeks before PTCA, because in a previous study (11), cholesterol levels remained stable thereafter. This resulted in an increase in serum cholesterol from 69.4 ± 19.4 mg/dl at baseline to 211.3 ± 42.6 mg/dl at the time of PTCA (n = 22).

Effect of the AP-1 consensus dODN on ET-1 synthesis. In a separate series of experiments, immunohistochemical analysis of coronary arteries harvested at 2, 3, 4, 7, 14 and 28 days after stent placement (n = 3 per time point) revealed positive ET-1 staining, beginning at day 2 and reaching a maximum at day 4; after 28 days, all segments stained negative (A. B. Buchwald, unpublished observation). Hence, ET-1 synthesis in this model starts early after PTCA and subsides after approximately two weeks.

As shown in Figure 1, in AP-1 consensus dODN-treated segments, ET-1 was hardly detectable 48 h after PTCA. In contrast, there was a distinct ET-1 immunoreactivity in vehicle-treated segments, showing particularly intense staining in the periluminal cell layers.

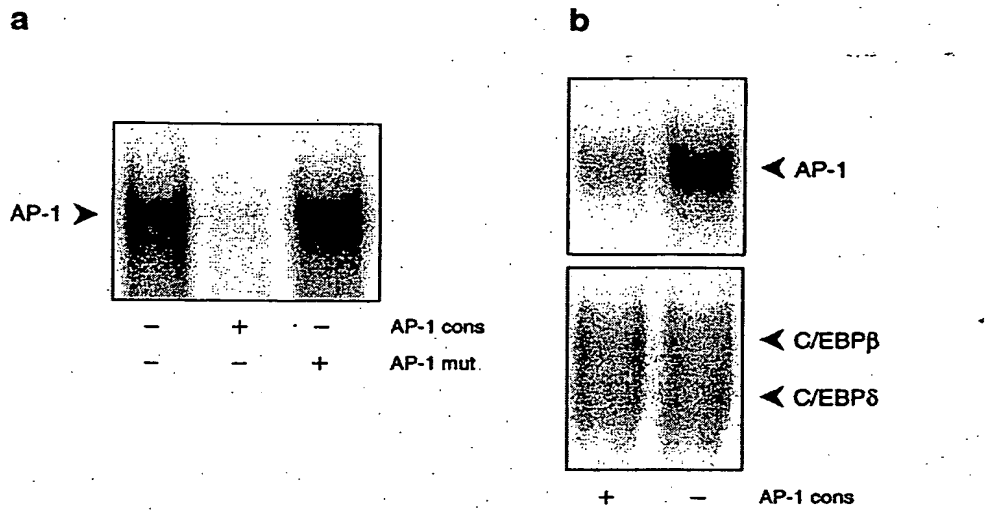

Figure 2. (a) The effects of pretreatment (1 h) with activator protein-1 (AP-1) consensus (AP-1 cons) or AP-1 mutant (AP-1 mut) decoy oligodeoxynucleotide (dODN) (10 μmol/l each) on phorbol dibutyrate (1 μmol/l)-induced nuclear translocation of AP-1 in endothelium-intact segments of the porcine left anterior descending coronary artery from the same animal. The segments were incubated in Waymouth medium containing 10% fetal bovine serum for 1 h at 37°C, followed by electrophoretic mobility shift analysis. The results shown are representative of at least two further experiments with segments from different animals. (b) In vivo treatment of porcine coronary arteries from the same animal, with AP-1 consensus dODN resulting in a diminished nuclear translocation of AP-1 relative to the buffer control 48 h after percutaneous transluminal coronary angioplasty (upper box), although nuclear translocation of CCAAT/enhancer binding protein (C/EBP)β and -δ was not affected. The results shown are representative of those obtained with four different animals. Identification of the C/EBP family members was done by supershift analysis (10).

Transcription factor activation. Electrophoretic mobility shift analysis revealed that the AP-1 consensus dODN completely prevented nuclear translocation of AP-1 in endothelium-intact, in vitro porcine coronary artery segments that had been exposed to 1 μmol/l of phorbol dibutyrate to upregulate AP-1 activity through stimulation of protein kinase C (Fig. 2a). In contrast, the AP-1 mutant dODN did not affect AP-1 abundance in the nucleus under these conditions.

Treatment of the coronary arteries with AP-1 consensus dODN in vivo resulted in a markedly reduced AP-1 activity in the vessel wall 48 h after PTCA, compared with the vehicle-treated coronary artery from the same animal (Fig. 2b). Treatment with AP-1 dODN, in contrast, had no effect on the nuclear translocation of C/EBPβ and -δ, two deformation-sensitive members of the C/EBP family of transcription factors (10).

Proliferative vessel wall response with dODN treatment. Injury scores in AP-1 consensus and AP-1 mutant dODN-treated arteries (range 2.0 to 2.1) were comparable to those in the buffer control group (Table 1). Figure 3 displays the morphometric analysis of a typical experiment with three different coronary arteries from the same animal treated with either vehicle, AP-1 consensus dODN or AP-1 mutant dODN. Of note, the AP-1 consensus dODN treatment had a pronounced effect on neointimal proliferation, despite comparable injury in all three arteries. After four weeks of follow-up, the minimal lumen area was clearly larger and the neointimal area was significantly smaller in AP-1 consensus dODN-treated segments than in vehicle-

Table 1. Histologic and Morphometric Analysis of the Effects of the Different dODN in the Coronary Angioplasty Model

	Buffer (n = 10)	AP-1 cons (n = 12)	AP1-21 mut (n = 7)	C/EBP cons (n = 3)	C/EBP mut (n = 3)	AP-1 cons (n = 3)	AP-1 mut (n = 3)
Follow-up (weeks)	4	4	4	4	4	8	8
Injury score	2.07 ± 0.06*	2.08 ± 0.04	2.03 ± 0.03	2.34 ± 0.19	2.27 ± 0.14	1.99 ± 0.02	2.09 ± 0.06
Minimal lumen area (mm^2)	3.78 ± 0.70†	5.61 ± 0.63	4.78 ± 0.74	2.39 ± 0.95	4.62 ± 0.88	2.78 ± 0.57	3.38 ± 0.48
Percent area stenosis	86.2 ± 9.1†	32.5 ± 2.2	49.4 ± 6.3	87.6 ± 8.0	69.7 ± 7.6	43.4 ± 2.3	69.5 ± 11.5
Medial area (mm^2)	1.72 ± 0.19*	1.89 ± 0.21	2.12 ± 0.17	2.01 ± 0.04	2.34 ± 0.15	1.63 ± 0.05	1.30 ± 0.19
Adventitial area (mm^2)	2.57 ± 0.59*	1.50 ± 0.27	3.14 ± 0.81	4.77 ± 0.92	5.37 ± 0.73	1.15 ± 0.14	1.58 ± 0.21
Total vessel area (mm^2)	14.10 ± 1.58*	12.32 ± 1.19	15.22 ± 1.35	19.68 ± 2.14	20.49 ± 1.61	8.00 ± 0.75	9.87 ± 0.32

*p = NS versus all other groups. †p < 0.05 versus AP-1 cons. Data are presented as the mean value ± SEM, except for follow-up. n denotes the number of coronary arteries analyzed (mean of 3 sections/stent) per treatment group. Note that due to destruction of 1 buffer control-treated segment before embedding in methylmethacrylate, the number of segments in this group is 10 instead of 11.

AP-1 = activator protein-1; AP-1 cons = AP-1 consensus dODN; AP-1 mut = AP-1 mutant dODN; C/EBP = CCAAT/enhancer binding protein consensus dODN; C/EBP mut = C/EBP mutant dODN; dODN = decoy oligonucleotide.

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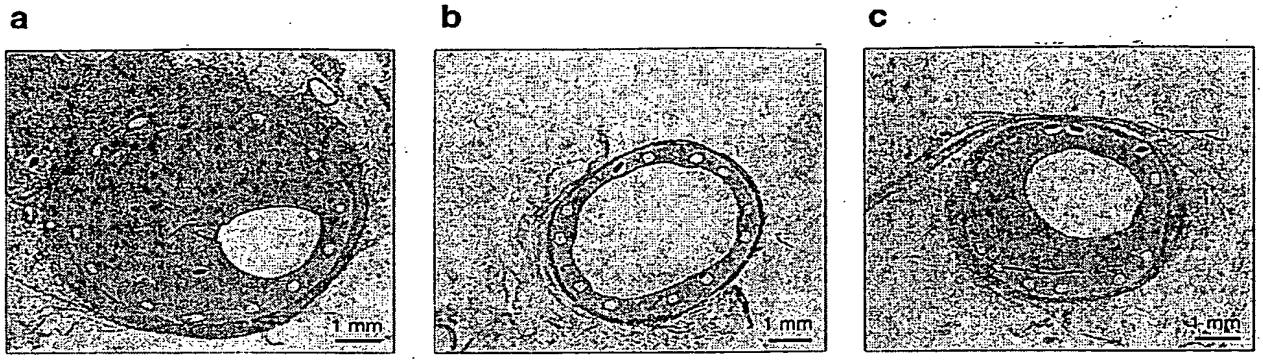

Figure 3. The proliferative vessel wall response four weeks after stent angioplasty in three coronary arteries from the same animal infused with vehicle (a), activator protein-1 (AP-1) consensus decoy oligodeoxynucleotide (dODN) (b) or AP-1 mutant dODN (c). Extensive proliferation in (a) and (c) is markedly reduced in the AP-1 consensus dODN-infused artery (elastica–van Gieson staining, original ×40, before 00% reduction).

treated or AP-1 mutant dODN-treated segments (Fig. 4). The media and total vessel area were comparable between the treatment groups. There was no evidence of decreased cellularity in the media in any of the treatment groups, as an indicator of potential toxic induction of fibrosis or calcification (Table 1).

This effect of AP-1 consensus dODN, as well as its effects on the adventitial, luminal (Table 1) and neointimal area (Fig. 5a), were maintained at eight weeks after PTCA, compared with AP-1 mutant dODN. No comparison with the buffer control group was made at this point in time, as previous studies with the minipig model have shown that neointimal formation manifests at four weeks and does not progress or regress over the following eight weeks (11).

To confirm that AP-1 consensus dODN exerts a specific effect that cannot be mimicked by a dODN directed against another transcription factor that is also deformation-sensitive and involved in the regulation of ppET-1 expression in another species (rabbit carotid artery [10]), the effects of C/EBP consensus dODN on restenosis four weeks after PTCA were investigated as well. However, both the consensus and corresponding mutant dODN did not inhibit neointimal formation, compared with vehicle-treated segments (Table 1, Fig. 5b).

DISCUSSION

Study findings. The main findings obtained in the minipig angioplasty model can be summarized as follows. Infusion of dODN against the transcription factor AP-1, using a local drug delivery catheter, results in a profound decrease in AP-1 activity and marked suppression of intravascular ET-1 synthesis 48 h after PTCA. This, in turn, contributes to a significant reduction of neointimal proliferation as the major determinant of in-stent restenosis, compared with buffer as the control, by >50% after four weeks. The corresponding mutant dODN, in contrast, has no effect. Moreover, inhibition of proliferation is maintained after eight weeks, indicating a definitive effect, not merely a delay of proliferation, occurring later than that in control arteries. The effect is achieved by a single application of the "naked" dODN at the time of PTCA, with no further treatment.

Role of ET-1 in in-stent restenosis. The action of ET-1 has been previously suggested as an important contributor to

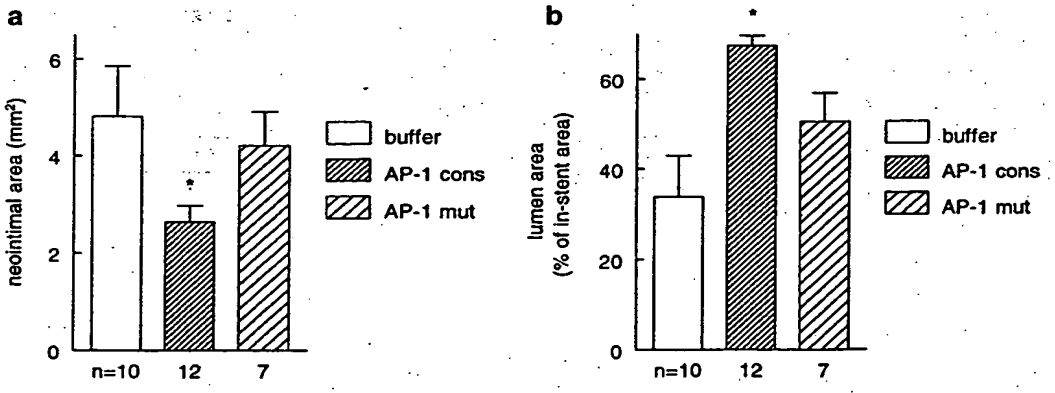

Figure 4. A comparison of the effects of activator protein-1 (AP-1) consensus (AP-1 cons) decoy oligodeoxynucleotide (dODN), AP-1 mutant (AP-1 mut) dODN and buffer control on neointimal area (a) and lumen area (b), expressed as the percentage of the in-stent area four weeks after percutaneous transluminal coronary angioplasty. *$p < 0.05$ vs. buffer control. †$p < 0.05$ vs. AP-1 mutant dODN.

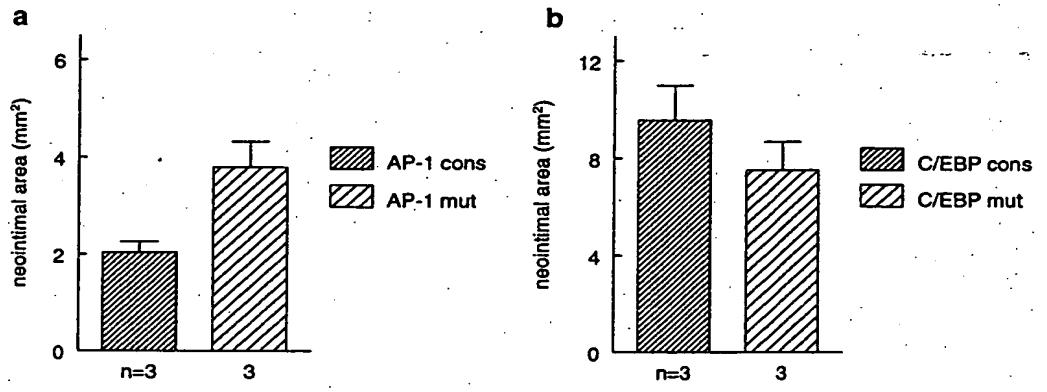

Figure 5. (a) A comparison of the effects of activator protein-1 (AP-1) consensus (AP-1 cons) and AP-1 mutant (AP-1 mut) decoy oligodeoxynucleotide (dODN) on neointimal area eight weeks after percutaneous transluminal coronary angioplasty (PTCA). (b) A lack of effect of C/EBP consensus (C/EBP cons) and C/EBP mutant (C/EBP mut) dODN on neointimal area four weeks after PTCA (see Fig. 4a for comparison).

post-PTCA restenosis. Tissue ET-1 levels increase after endothelial denudation in a rabbit carotid artery model (8), and ET-1 is released from human atherosclerotic coronary arteries after injury (9). Selective antagonism of the endothelin A (ET_A) receptor reduced the proliferative vessel wall response in a rat carotid artery (15) and a porcine femoral and carotid artery PTCA model (16), in accordance with the findings of this study. Similarly, the approach of selective ET_A receptor antagonism in a non-hypercholesterolemic porcine coronary stent angioplasty model reduced neointimal hyperplasia (17). However, a successful reduction in proliferation was observed, with modest proliferation in the control/placebo groups (e.g., average neointimal thickness of 0.45 mm in the study by McKenna et al. (17) vs. 1.03 mm in the present study). In contrast, in this study, inhibition of ET-1 synthesis by the AP-1 dODN limited neointimal proliferation, irrespective of the degree of arterial injury. Moreover, this effect was achieved with a single periprocedural local infusion of dODN, while antagonizing the receptor-required systemic administration of the drug over several days.

Vessel wall trauma and therapeutic efficacy. The proliferative vessel wall response increases with increasing stretch, and thus injury to the vessel wall with PTCA (12,13). In accordance with the available evidence suggesting stretch-induced activation of ET-1 synthesis as a major determinant of the proliferative vessel wall response to injury, exceeding the adaptive proliferation secondary to mild stretch, the results of the present study support the hypothesis that inhibition of ppET-1 gene expression and, consequently, the synthesis of mature ET-1 results in a limited proliferative response, even in areas of deep vessel wall injury.

Specificity of the dODN approach. It could be argued that any mechanosensitive transcription factor can cause neointimal proliferation after PTCA, especially if this transcription factor is potentially associated with the expression of the target gene, too (10). However, the negative results obtained in the animals treated with the C/EBP dODN indicate that the proliferative response in this model is specifically mediated by AP-1. This does not mean, however, that the inhibitory effect of AP-1 dODN on in-stent restenosis is solely due to the blockade of deformation-induced ppET-1 expression. Other gene products involved in neointimal formation, the expression of which is influenced by AP-1, may be likewise affected, although from a therapeutic point of view, this does not necessarily pose a problem.

Clinical perspective. Given that the aforementioned results can be extrapolated to the situation in patients with coronary artery disease, the observed extent of the inhibitory action of dODN treatment suggests that this co-medication may greatly reduce the incidence of in-stent restenosis, which at present ranges from 10% to 40% after six months.

Study limitations. A potential limitation of this study is that it was performed in minipigs, and although these animals were fed an atherogenic diet, PTCA was performed in near-healthy coronary arteries. In the clinical situation, severe atherosclerosis and calcification, replacing parts of the normal vessel wall, may influence the role of the endothelin system in the reparative response after PTCA (9). Although the release of ET-1 from human coronary arteries after PTCA suggests a role for the peptide in human atherosclerosis, as well, the dODN approach must be tested in a clinical setting.

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